RESEARCH ARTICLE

Protein synthesis is lowered by 4EBP1 and eIF2-α signaling while protein degradation may be maintained in fasting, hypoxic Amazonian cichlids *Astronotus ocellatus*

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ABSTRACT

The Amazonian cichlid *Astronotus ocellatus* is highly tolerant to hypoxia, and is known to reduce its metabolic rate by reducing the activity of energetically expensive metabolic processes when oxygen is lacking in its environment. Our objectives were to determine how protein metabolism is regulated in *A. ocellatus* during hypoxia. Fish were exposed to a stepwise decrease in air saturation (100%, 20%, 10% and 5%) for 2 h at each level, and sampled throughout the experiment. A flooding dose technique using a stable isotope allowed us to observe an overall decrease in protein synthesis during hypoxia in liver, muscle, gill and heart. We estimate that this decrease in rates of protein synthesis accounts for a 20 to 36% decrease in metabolic rate, which would enable oscars to maintain stable levels of ATP and prolong survival. It was also determined for the first time in fish that a decrease in protein synthesis during hypoxia is likely controlled by signaling molecules (4EBP1 and eIF2-α), and not simply due to a lack of ATP. We could not detect any effects of hypoxia on protein degradation as the levels of NH_4 excretion, indicators of the ubiquitin proteasome pathway, and enzymatic activities of lysosomal and non-lysosomal proteolytic enzymes were maintained throughout the experiment.

KEY WORDS: Hypoxia, Protein synthesis, Protein degradation, Oscars, Signaling pathways

INTRODUCTION

The cichlid *Astronotus ocellatus* (Agassiz 1831), commonly known as the oscar, is one of the most hypoxia-tolerant species in the Amazon (Almeida-Val et al., 1993) and as such, is an excellent model for studying mechanisms of hypoxia tolerance (Lewis et al., 2007). When oxygen saturation decreases, fish undergo several physiological and behavioral modifications in order to prolong survival (reviewed in Farrell and Richards, 2009; Richards, 2010). Such modifications include an increase in gill ventilation, gill remodeling, and increased cardiac output (Farrell and Richards, 2009; Richards, 2010). When oxygen levels drop below a certain threshold, oxygen consumption rates decrease linearly with decreasing oxygen concentration (Rogers et al., 2016). At this

stage, hypoxia-sensitive animals maintain low levels of activity fueled by anaerobic metabolism (Boutilier, 2001), while hypoxia-tolerant fish suppress their metabolic rate to conserve energy and limit accumulation of toxic end-products, such as lactate, to prolong survival (Boutilier, 2001; Richards, 2009). Different species have varying capacities to reduce their metabolic rate (Rogers et al., 2016). Oscars decrease their metabolic rate by 30% and 50% of resting metabolic rate at air saturation levels of 20% and 10%, respectively (Lewis et al., 2007; Muusze et al., 1998). Adjustments in metabolic rate consist of suppressing processes that require high energy expenditure such as reproduction, digestion and protein synthesis (Richards, 2009). Protein synthesis is considered one of the most energetically demanding metabolic processes, requiring approximately 20–40% of total oxygen consumption (Carter et al., 1993; Houlihan et al., 1988). However, there is no consensus for the exact cost of protein synthesis in fish, and the cost is likely to vary among tissues, species and developmental stages (Houlihan et al., 1995).

Even though fish are frequently exposed to hypoxia, only limited information is available regarding the molecular mechanisms responsible for the hypoxia response, especially concerning protein metabolism. This aspect is mostly studied in mammalian cell lines (Wouters and Koritzinsky, 2008). However, recent studies on fish are beginning to show similar regulatory processes. Three of the major signaling pathways that are involved in the hypoxia response are HIF (hypoxia inducible factor), mTOR (mechanistic target of rapamycin) and the UPR (unfolded protein response) (Wouters and Koritzinsky, 2008). Under optimal growth conditions, mTOR is activated and, amongst other actions, phosphorylates a downstream target, 4EBP1, resulting in an increase of protein synthesis (Johnston et al., 2011). During hypoxia, mTOR is inhibited through multiple means, which in turn inhibits protein synthesis (Wouters and Koritzinsky, 2008). HIF regulates mTOR during hypoxia by activating the TSC1–TSC2 complex, which directly inhibits mTOR activity in mammalian cells (Wouters and Koritzinsky, 2008). AMPK, an adenylate energy charge sensor, is activated during hypoxia in common killifish (*Fundulus heteroclitus*) and common carp (*Cyprinus carpio*) (Hallman et al., 2008; Richards et al., 2008), which also activates the TSC1–TSC2 complex (Liu et al., 2006). Activation of any of these pathways could lead to a decrease in the level of phosphorylated 4EBP1 and, consequently, a decrease in rate of protein synthesis. A second major means of control of protein synthesis is via eIF2-α. Under hypoxic conditions, PERK (protein kinase RNA-like endoplasmic reticulum kinase) is activated and phosphorylates eIF2-α, thus blocking translation initiation and protein synthesis (Koritzinsky et al., 2006; Koumenis et al., 2002; Ron and Walter, 2007).

Protein degradation is an important energy-consuming process that has seldom been studied in fish exposed to hypoxic conditions.

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List of abbreviations

4EBP1	eukaryotic translation initiation factor 4E-binding protein 1
AKT	protein kinase B
AMPK	AMP-activated protein kinase
ATF4	activating transcription factor 4
eIF2AK3	eukaryotic initiation factor-2α subunit kinase
eIF2-α	eukaryotic initiation factor 2-alpha
HIF	hypoxia inducible factor
IGF	insulin-like growth factor
mTOR	mechanistic target of rapamycin
p70S6K	ribosomal protein S6 kinase beta-1
PERK	protein kinase RNA-like endoplasmic reticulum kinase
TSC1–TSC2	hamartin–tuberin complex
UPR	unfolded protein response

Fig. 1. Experimental setup. Oscars were exposed to a stepwise decrease in air saturation levels (100%, 20%, 10% and 5%) for 2 h at each level. Five fish were sampled at each level, having spent 2 h at each previous air saturation level. The first group of control fish remained at 100% air saturation for 2 h. Another group of control fish remained at 100% air saturation for 8 h. Fish were injected with deuterated phenylalanine 2 h prior to sampling (represented by the syringe symbols).

One of the major protein degradation pathways is the ubiquitin–proteasome pathway, in which proteins are targeted for degradation via binding of a polyubiquitin chain in an ATP-requiring reaction involving three complex groups of enzymes (E1, E2 and E3) (Ciechanover et al., 1984; Hershko and Heller, 1985). The polyubiquitin chain is then recognized by the proteasome complex, which proceeds to degrade the target protein and recycle the ubiquitin moieties (Ciechanover et al., 1984). Protein degradation may also occur via proteolysis in the lysosome catalyzed by low pH-active cathepsins and non-lysosomal intracellular calcium-dependent calpains. In mammals, the lysosomal pathway is activated by low cytosolic ATP during hypoxia (Glick et al., 2010). Here, we report 20S proteasome activity, relative quantity of polyubiquitinated protein, and activity of cathepsin and calpain proteases to assess whether these markers of protein degradation are altered by hypoxia as a means of decreasing energy demand.

This study focuses on the regulatory mechanisms of protein synthesis and protein degradation in the Amazonian cichlid *A. ocellatus* exposed to severe acute hypoxia. Standard lactate, glycogen and glucose analyses were conducted to assess the degree of anaerobic metabolism, as well as rates of NH$_4$ release to gauge rates of protein breakdown and amino acid utilization. The important novel contribution of this study is that it shows protein synthesis is decreased via cell signaling proteins such as 4EBP1 (mTOR pathway) and eIF2-α (UPR pathway) in fish exposed to hypoxic conditions in various tissues. We also provide evidence that suggests that under some situations protein degradation is not affected by acute hypoxic conditions in this species.

MATERIALS AND METHODS
Animals and experimental protocol
Adult oscars (*A. ocellatus*) (*N*=37; body mass=281±6.6 g; length=17±0.2 cm; values expressed as means±s.e.m.) were collected in mid-January 2016 by local fishermen. Fish were transferred to the Laboratory of Ecophysiology and Molecular Evolution at the Brazilian National Institute for Research in the Amazon, Manaus, Brazil, and held in running water at 28°C in an outdoor tank. Fish were offered food but declined to eat. Experiments were initiated 2 weeks after capture and extended for 7 days.

Fish were subjected to a hypoxic challenge (Fig. 1). Five fish were taken daily from the main holding tank and transferred to five glass aquaria containing 20 liters of fresh water. Two tanks were maintained at 100% air saturation; these fish were considered as controls and were sampled 2 and 8 h after the transfer. The other
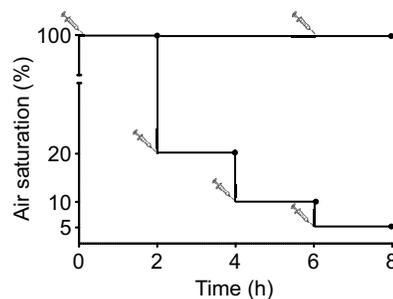
three tanks were subjected to a decrease in air saturation. Air saturation was decreased to 20% by displacing oxygen using nitrogen gas and after 2 h one fish was sampled. Thereafter, air saturation was decreased to 10% and again after 2 h one fish was sampled. Finally, air saturation was decreased to 5% and the final hypoxic treated fish was sampled 2 h later. Oxygen concentration was monitored using an oxygen meter (5512-Ft, YSI, Yellow Springs, OH, USA) and maintained at the required level of hypoxia by manually controlling the level of either nitrogen or air. The experiment was repeated five times with treatment being rotated through each of the five tanks to eliminate any potential tank effect. At the end of each hypoxic challenge, fish were killed by severing the spinal cord immediately behind the brain, and samples of liver, heart, white muscle, gill and blood were immediately collected. Blood samples were spun for 5 min at 2000 *g* to separate the plasma. All tissues were immediately frozen in liquid nitrogen and stored at −80°C for future analyses.

All experiments were conducted in accordance with Brazilian law under the project title 'Centre for Studies of Adaptations of Aquatic Biota of the Amazon ADAPTA-AMAZONIA' and permit number 29837-9 authorized by Comissão de Ética no Uso de Animais - Instituto Nacional de Pesquisas da Amazônia (CEUA-INPA).

Lactate and glucose
Plasma, muscle and liver were analyzed for lactate and glucose. Tissues were deproteinized with 6% perchloric acid and neutralized with 2 mol l^{-1} KHCO$_3$. Lactate was analyzed in glycine buffer containing NAD$^+$ and thereafter treated with excess lactate dehydrogenase. Glucose was analyzed in buffer containing 250 mmol l^{-1} imidazole, 5 mmol l^{-1} MgSO$_4$, 10 mmol l^{-1} ATP, 0.8 mmol l^{-1} NADP$^+$ and excess glucose 6-phosphate dehydrogenase, and thereafter treated with excess hexokinase. Standard curves were created for lactate and glucose following the same procedures for tissue analysis. All assays were conducted using a plate reader (SpectraMax M, Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 340 nm. Glycogen was assessed in glucosyl units following amyloglucosidase treatment (Keppler and Decker, 1974).

Protein synthesis
The fractional rate of protein synthesis was measured using the flooding dose technique by Garlick et al. (1980) and modified to use stable isotopes (Cassidy et al., 2016; Lamarre et al., 2015). The technique was first validated for this species by injecting fish

intraperitoneally with a solution of 150 mmol l^{-1} phenylalanine solution containing 50% [D$_5$]-L-phenylalanine (ring-[D$_5$]-PHE, 98%, Cambridge Isotope Laboratories, Andover, MA, USA) at a dosage of 0.6 ml per 100 g of fish body mass. The injection procedure was conducted without anesthesia and took less than 1 min to complete. Thereafter, animals were sampled immediately or after 1, 2 or 3 h with three animals selected for each time point to determine the appropriate incorporation period. We determined that 2 h was the optimal incorporation period (see Results). Fish used in the hypoxia experiment received the isotope injection immediately after adjusting air saturation, and were returned to their experimental tank for 2 h prior to sampling. The fish were then killed as above and the tissues immediately frozen in liquid nitrogen.

The fractional rate of protein synthesis was measured as described in Lamarre et al. (2015) by first weighing 75 mg of tissue and homogenizing in 1 ml of 0.2 mol l^{-1} perchloric acid using a sonicating homogenizer (Q55 Sonicator, Qsonica). After being centrifuged at 15,000 g for 5 min at 4°C, the supernatant, which contains the free amino acid pool, was transferred into a clean labeled tube and frozen. The protein pellet was washed three times in 1 ml of 0.2 mol l^{-1} perchloric acid and then washed once in acetone before being hydrolyzed in 6 ml HCl at 110°C for 18 h. The phenylalanine was extracted using solid phase extraction (Bond-Elut C-18, Varian). The extracted samples were dried and then derivatized using pentafluorobenzyl bromide as an alkylating agent (Cassidy et al., 2016; Lamarre et al., 2015).

The [D$_5$]-PHE enrichment of the free phenylalanine pool and protein pool were measured by GC-MS. The system was composed of an Agilent gas chromatograph (model 7890B) interfaced with a single quadrupole mass selective detector (MSD 5977B). The chromatographic conditions were as described elsewhere (Lamarre et al., 2015). Peak detection and integration was performed using MassHunter (Version B07.01 SP2, Agilent).

The fractional rate of protein synthesis (K_s in % day^{-1}) was calculated using the formula:

$$K_s = \frac{S_b}{S_a} \times \frac{1440}{t} \times 100, \qquad (1)$$

where S_b is the enrichment of the protein pool, S_a is the enrichment of the free amino acid pool, t is the incorporation time (min) and 1440 is the conversion from minutes to days (Lamarre et al., 2015).

Levels of phosphorylation

The levels of phosphorylated proteins were measured by western blots. Tissues were homogenized in nine volumes of lysis buffer (50 mmol l^{-1} Tris, 0.1 mmol l^{-1} EDTA, 1.0 mmol l^{-1} β-mercaptoethanol, pH 8) using a sonicating homogenizer and centrifuged at 13,000 g for 10 min at 4°C, and the supernatant was collected. Protein concentration was measured using the Bradford protein assay (Bio-Rad, Hercules, CA, USA) (Bradford, 1976). Tissue homogenates were subjected to SDS-PAGE and blotted on PVDF membranes. The membranes were blocked with 5% BSA and then probed with protein-specific antibodies from Cell Signaling Technologies (Beverley, MA, USA): 4EBP1 (no. 9649), p-4EBP1 (thr 37/46; no. 2855), eIF2-α (no. 5324) and p-eIF2-α (ser51; no. 3398). The bands were revealed by probing with a goat anti-rabbit HRP-conjugated antibody (Cell Signaling Technologies). The membranes were revealed using enhanced chemiluminescence (ECL) and imaged using ECL-sensitive films (Amersham Hyperfilm ECL, GE Life Science, São Paulo, Brazil).

The films were developed using autoradiography developers and fixer (Carestream, Kodak GBX, Rochester, NY, USA), scanned (DCP-7065DN, Brother) and the densitometric analysis was performed using Image Lab 5.2.1 software (Bio-Rad). Levels of phosphorylation were obtained by calculating the ratio between phosphorylated and total proteins (e.g. phospho-4EBP1/total 4EBP1).

20S proteasome activity

Chymotrypsin-like activity of the 20S proteasome was measured as described in Lamarre et al. (2012). Tissues were homogenized as described for the western blot analyses. 20S proteasome activity was measured by adding 50 µg of protein in wells of black-bottomed 96-well plates in quadruplicate along with 100 µl of assay buffer (100 mmol l^{-1} Tris, 0.0475% SDS, pH 8) and 10 µl of the synthetic fluorogenic substrate LLVY-AMC (Enzo Life Sciences, Burlington, ON, Canada, p802-0005; 400 µmol l^{-1} in Tris buffer). In the fourth replicate of each sample, an inhibitor solution of the 20S proteasome (ZLLL-CHO, Enzo Life Sciences, PI102-0005) was added. Fluorescence was read at excitation/emission wavelengths of 370/430 nm with a multi-mode microplate reader (SpectraMax M, Molecular Devices). The inhibitor sensitive activity of the 20S proteasome was expressed in arbitrary fluorescent units per minute, per 50 µg of protein.

Levels of polyubiquitinated proteins

Dot blot analyses were used to determine the relative levels of polyubiquitinated proteins in the fish tissues. The protocol used is similar to that described in Cassidy et al. (2016) and Lamarre et al. (2012). Aliquots of the tissue homogenates from the 20S proteasome assay were used for dot blot analyses. Approximately 25 µg of protein of each sample was spotted on a nitrocellulose membrane. The membrane was blocked with 5% BSA, a monoclonal antibody specifically detecting polyubiquitinated conjugates (mAB, FK1, Enzo, BML-PW8805) and an anti-mouse IgM HRP-linked antibody (ab97230, Abcam) was used to detect polyubiquitinated proteins. The membranes and films were treated as described above.

Cathepsin and calpain activity

The cathepsin and calpain activities were determined in the same homogenate used for the proteasome assay (above). Cathepsin A-, B-, H- and L-like activity was determined at pH 5.5 (97 mmol l^{-1} citric acid, 5.8 mmol l^{-1} Na2HPO4 and 0.1% mercaptoethanol). The calpain activity was determined at pH 7.5 (20 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, 10 mmol l^{-1} CaCl2, 100 mmol l^{-1} KCl and 0.1% mercaptoethanol). In all cases, the chromogenic substrate was azocasein at a final concentration of 10.4 mg ml^{-1} and the assay was performed as described in Barrett and Kirschke (1981). The assay was stopped after 60 min by adding 200 µl of 5% trichloroacetic acid in water and the samples were spun at 20,000 g for 5 min. Two-hundred microliters of the supernatant was then transferred into a microplate and 50 µl of 1 mol l^{-1} NaOH was added to each well. The absorbance was immediately read at 440 nm.

Ammonia excretion

NH$_4$ in water was assayed by the method of Verdouw et al. (1978). Standard curves of NH$_4$ (as NH$_4$SO$_4$) were prepared up to 50 µmol l^{-1} in milliQ water. All samples were analyzed in triplicate and fell within the linear range of the assay. NH$_4$ was assayed in water samples at time zero and at 30 min intervals thereafter, for all fish that were injected with phenylalanine. Trend lines were calculated for the 2 h period.

Statistical analyses

Values are expressed as means±s.e.m. One-way ANOVAs with Tukey's *post hoc* tests were performed to assess the effects of the different air saturation levels on the variable measured. Box–Cox transformations were applied when needed to improve normality of residuals. A *P*-value smaller than 0.05 was considered statistically significant. Data Desk 6.3 (Data Description Inc., Ithaca, NY, USA) was used for the statistical analyses while Prism 7 (GraphPad Software, La Jolla, CA, USA) was used to format the figures.

RESULTS

There were no mortalities during the experiment and the fish did not lose equilibrium, even after 2 h at 5% air saturation.

Lactate and glycogen

Lactate concentration was measured in plasma, liver and muscle of fish exposed to different levels of air saturation (Fig. 2). Lactate in plasma was elevated in the initial group sampled at 100% air saturation and following severe hypoxia. Lactate in liver significantly increased at a hypoxic challenge of 5% air
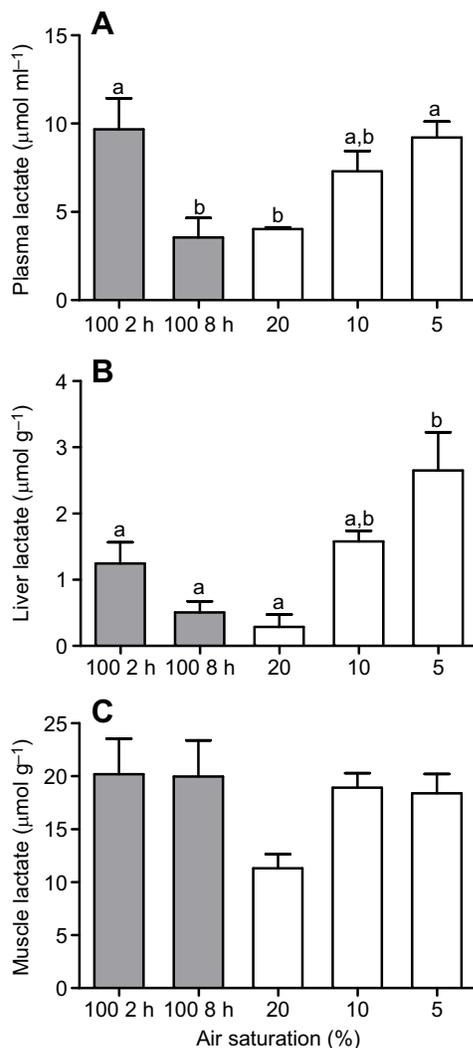
saturation. There was no change in lactate levels in muscle that remained at approximately 18 µmol g^{-1}.

Glucose concentration was measured in plasma and glycogen content was determined in liver and muscle. Plasma glucose remained constant across experimental groups with an overall value (all groups considered) of 10.0±0.64 µmol ml^{-1} ($N=25$; data not shown). Free glucose in liver and muscle was always below 0.12 µmol g^{-1} (data not shown). Liver glycogen levels remained constant with mean values between 160 and 188 µmol glycosyl units g^{-1}. Similarly muscle glycogen did not change with treatment, with mean values ranging between 8.9 and 15 µmol glycosyl units g^{-1} (Fig. 3).

Protein synthesis
Validation of the protein synthesis assay

The flooding dose technique is only valid when the assumptions are verified (Fraser and Rogers, 2007; Lamarre et al., 2015). The first assumption is that the injected D_5-PHE rapidly floods the tissues and the second assumption is that the enrichment level of D_5-PHE in the free amino acid pool is stable for the duration of the experiment. Although the enrichment level of the free pool in the liver appears to decrease (Fig. 4A), the linear regression failed to reach the significance level ($P=0.07$). The first two assumptions are therefore met. Following the injection, the tracer flooded the tissues, reaching ~35% enrichment in both the liver and the muscle, and remained stable for up to 180 min (Fig. 4A,B). The last assumption is that the incorporation of the tracer in the protein pool increases with time in a linear fashion. This assumption was also met, as can be seen in Fig. 4C,D. From these results, we selected an incorporation period of 120 min in the following experiments.

Protein synthesis in oscars exposed to hypoxia

Fractional rates of protein synthesis (K_s) were measured in gill, heart, muscle and liver of oscars exposed to a stepwise decrease in

Fig. 2. Lactate levels in plasma, liver and muscle of oscars exposed to 100%, 20%, 10% and 5% air saturation. Values are expressed in µmol per gram of tissue (means±s.e.m., *n*=5). Mean values with the same lower case letters are not significantly different from each other (*P*>0.05).
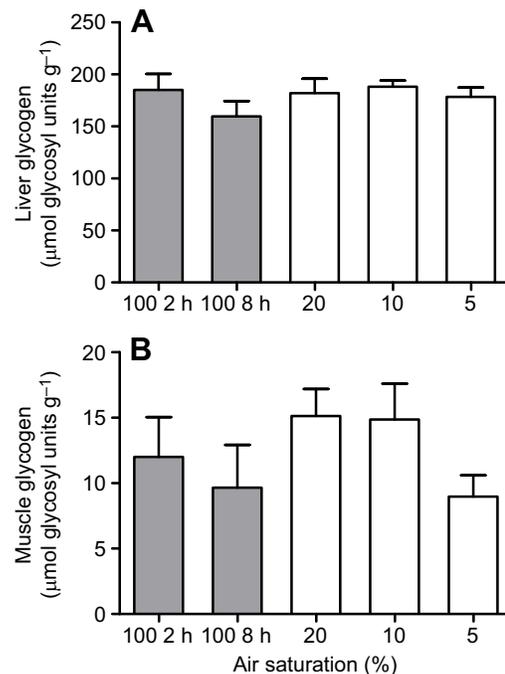

Fig. 3. Glycogen levels in liver and muscle of oscars exposed to 100%, 20%, 10% and 5% air saturation. (A) Liver; (B) muscle. Values are expressed in µmol per gram of tissue (means±s.e.m., *n*=5). There were no significant differences (*P*>0.05) between groups.
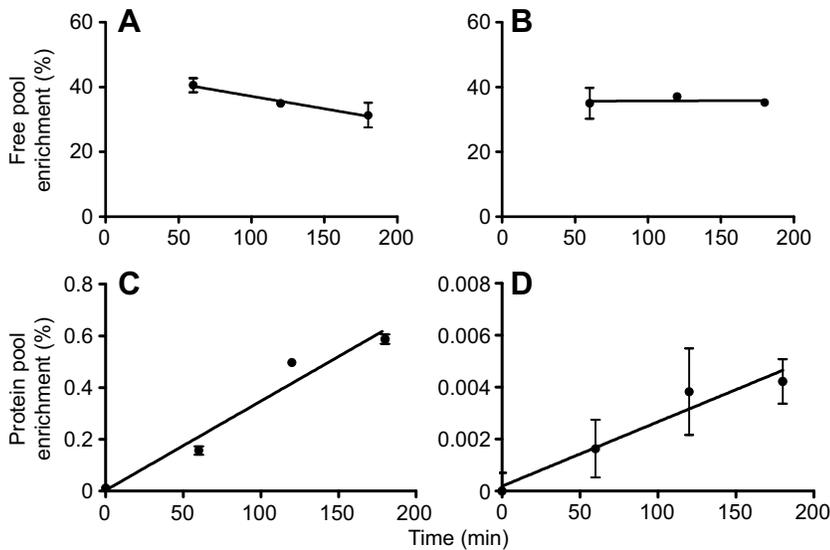
Fig. 4. Specific enrichment (%) of the free PHE pool and the protein pool of liver and white muscle in relation to time after injection of the tracer in *A. ocellatus*.
(A,B) free PHE pool of liver (A) and white muscle (B).
(C,D) Protein pool of liver (C) and white muscle (D). Values are expressed as means±s.e.m. (*n*=3).

air saturation levels (Fig. 5). Rates of protein synthesis in gills decreased at 10 and 5% air saturation relative to fish held at 100% air saturation. In heart and muscle there was a 40–70% decrease in K_s at 10 and 5% air saturation compared with controls. Liver showed the greatest decrease in rates of protein synthesis (86–96% decrease at 10 and 5% air saturation).

Phosphorylation levels of 4EBP1 (t37/46) were assessed in liver and heart. Phosphorylation levels of these tissues in fish exposed to 10 and 5% air saturation were significantly decreased compared with the control fish (100% air saturation for 8 h; Fig. 6). Phosphorylation levels of eIF2-α (ser51) were significantly increased in liver of fish at 10 and 5% air saturation, compared with the control fish (100% air saturation; Fig. 7). We attempted multiple times to measure phosphorylation levels of eIF2-α in the heart; however, we could not obtain a signal for either phosphorylated or total protein.

Protein degradation

The activity of 20S proteasome, cathepsins and calpains was unaffected by hypoxia in all tissues studied (Table 1). There was no significant effect of air saturation levels on polyubiquitinated proteins in heart and liver, and there was a transient decrease of polyubiquitinated proteins at 20% air saturation in gill (Table 1).

NH_4 excretion was quite variable under all air saturation conditions. Trend lines for NH_4 excretion were calculated for the 2 h period for each oxygen level. There was no significant difference in rates of NH_4 excretion with respect to air saturation, although the average rate was approximately twofold higher between 5% and 100%. More specifically, rates were 491± 153 µmol kg^{-1} h^{-1} (*n*=5) and 821±281 µmol kg^{-1} h^{-1} (*n*=5) at 5 and 100% air saturation, respectively.
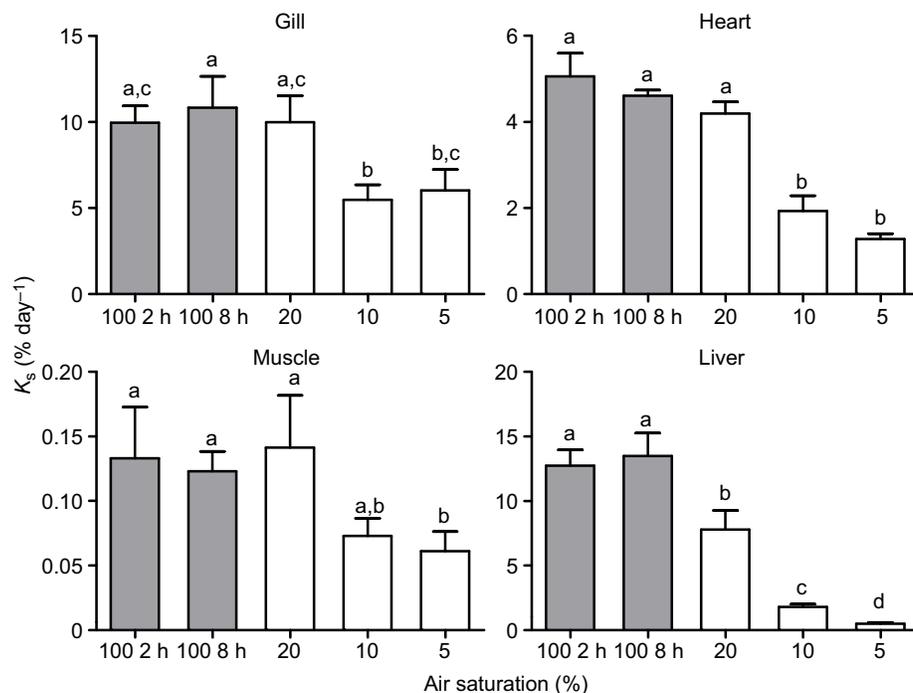

Fig. 5. Fractional rate of protein synthesis (K_s; mean±s.e.m., *n*=5) of gill, heart, muscle and liver in oscars exposed to a stepwise decrease in oxygen saturation levels (100, 20, 10 and 5% air saturation). Mean values with the same lowercase letters are not significantly different from each (*P*>0.05) other.
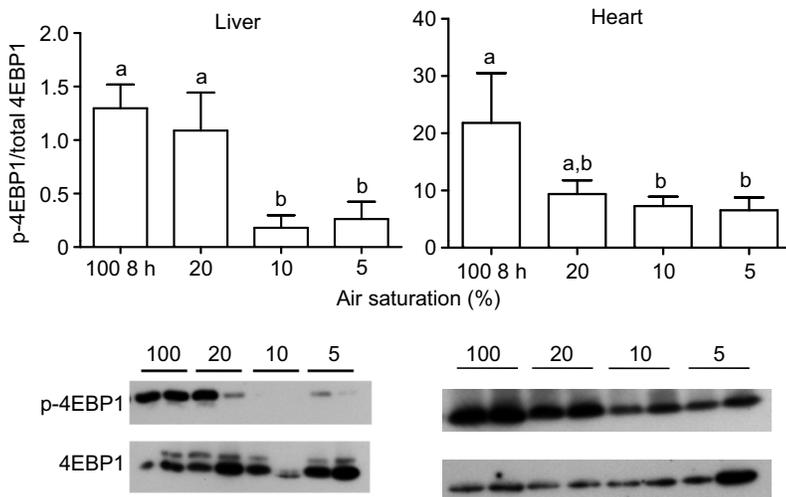
Fig. 6. Phosphorylation levels of 4EBP1 (T37/46) in liver and heart (mean±s.e.m., $n=5$) of oscars exposed to a stepwise decrease in oxygen saturation levels (100, 20, 10 and 5% air saturation). Data are expressed as ratios of phosphorylated protein over total protein. Mean values with the same lowercase letters are not significantly different from each ($P>0.05$) other. Representative blots are shown below.

DISCUSSION

Carbohydrate metabolism

Exposing oscars to a stepwise decrease in oxygen saturation levels allowed us to test various physiological responses to hypoxia. Lactate levels in plasma were higher in fish held in 100% air saturation and sampled 2 h after transfer to experimental chambers than in fish held for 8 h under control conditions, suggesting that the initial movement of fish induced an anaerobic response. Increased lactate concentration was noted in plasma at 10 and 5% air saturation compared with fish held at 100% air saturation for 8 h. An increase in plasma lactate under hypoxic conditions is well recognized in oscars and the closely related *Astronotus crassipinnis* (Chippari-Gomes et al., 2005; Lewis et al., 2007; Richards et al., 2007; Scott et al., 2008; Wood et al., 2007). It should be appreciated that plasma represents ~5% of fish mass. Thus, an increase in plasma lactate in a 100 g fish from 4 to 10 μmol ml^{-1} would result in a total increase of lactate of 30 μmol. Although the increase in lactate suggests there is an activation of anaerobic metabolism in some tissues, the lactate levels in themselves provide limited information regarding the magnitude of any increase in whole-body lactate production. In the

present experiment, there was no change in glycogen concentration under hypoxia in white muscle. Muscle glycogen levels reported here are in the same range as previous studies that also showed either no decrease under hypoxia (De Boeck et al., 2013; Richards et al., 2007) or in the case of *A. crassipinnis* an increase following severe hypoxia (Chippari-Gomes et al., 2005). We also found no change in lactate concentration under hypoxia as reported by Lewis et al. (2007). This conflicts with findings of others where hypoxia resulted in an increase in muscle lactate from 8 to 17 μmol g^{-1} (Wood et al., 2007) and from 2 to 6 μmol g^{-1} (Richards et al., 2007). Differences in findings are likely due to the level and length of hypoxia. Muscle lactate level noted here is higher than that reported by others (Lewis et al., 2007; Wood et al., 2007; Richards et al., 2007) for normoxic fish. A careful analysis of our data leads us to conclude that our observation of ~20 μmol g^{-1} under most conditions is correct. We have no explanation for this finding. It is possible that the simple transfer of fish from the holding tank to the experimental chamber resulted in an increase in lactate. Regardless, it is clear that a hypoxic challenge did not lead to a further increase in muscle lactate in the present study, consistent with the lack of change in glycogen level.

In the present study, there was an increase of lactate in liver but only at 5% air saturation and only to the level of 2.6 μmol g^{-1}. There was no change in liver glycogen, which remained at approximately 175 μmol glycosyl units g^{-1}. This response is similar to that reported by Richards et al. (2007), where there was no change in either liver lactate or glycogen levels (approximately 200 μmol glycosyl units g^{-1}) even after 20 h at 5% air saturation. Similarly, an acute hypoxic event (3 h at approximately 10% air saturation) had no effect on liver glycogen content (De Boeck et al., 2013). In *A. crassipinnis*, glycogen levels showed no change down to 13% air saturation and only a very small decrease after 8 h at 6% air saturation (Chippari-Gomes et al., 2005). These findings are collectively important because they suggest that in *Astronotus*, under even severe conditions of hypoxia, there is little or no activation of glycogenolysis in liver or in white muscle, the tissue that constitutes the highest proportion of the body mass. The considerable glycogen reserves in liver may be more related to long-term food deprivation as opposed to anaerobic metabolism (De Boeck et al., 2013).
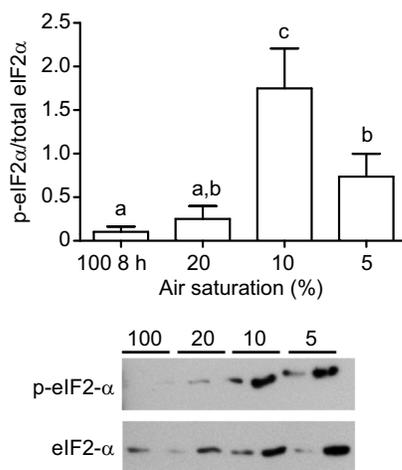

Fig. 7. Phosphorylation levels of eIF2-α (ser51) in liver (mean±s.e.m., $n=5$) of oscars exposed to a stepwise decrease in oxygen saturation levels (100, 20, 10 and 5% air saturation). Data are expressed as ratios of phosphorylated protein over total protein. Mean values with the same lowercase letters are not significantly different from each ($P>0.05$) other. Representative blots are shown below.

Protein synthesis

The fractional rate of protein synthesis was measured *in vivo* using a flooding dose technique with a stable isotope tracer (deuterated

Table 1. Protease activities and levels of polyubiquitinated proteins in heart, gill, liver and muscle of oscars exposed to different levels of air saturation

Tissue	Air saturation (%)	20S proteasome	Polyubiquitinated proteins	Cathepsins	Calpains
Heart	100		1.00±0.33		
	20		0.56±0.03		
	10		0.72±0.17		
	5		0.49±0.14		
Gill	100	1.00±0.18	1.00±0.08	1.00±0.05	
	20	0.99±0.19	0.60±0.07*	1.14±0.08	
	10	1.19±0.12	0.97±0.07	1.06±0.08	
	5	0.96±0.15	0.74±0.07	1.068±0.06	
Liver	100	1.00±0.11	1.00±0.08	1.00±0.23	
	20	1.05±0.05	0.77±0.08	1.50±0.27	
	10	0.89±0.05	1.02±0.06	1.11±0.21	
	5	1.06±0.04	0.88±0.10	1.18±0.44	
Muscle	100	1.00±0.10		1.00±0.05	1.00±0.42
	20	0.84±0.026		1.14±0.08	1.68±0.47
	10	0.96±0.03		1.06±0.08	0.99±0.19
	5	1.01±0.09		1.07±0.06	1.14±0.36

Values are expressed relative to the control group (100% air saturation) for each tissue (means±s.e.m., n=5). *Significant difference ($P<0.05$) from the control group.

phenylalanine) in several tissues during all levels of hypoxia exposure. All the validation criteria were met, giving assurance that the experimental approach was applicable. Under normoxic conditions, fractional rates of protein synthesis were such that liver>gill>heart>muscle. This sequence is similar to that reported by Lewis et al. (2007), who measured fractional rates of protein synthesis with radioactive [3H] phenylalanine. The power of the current, more sensitive approach allowed measurement of protein synthesis in white muscle that Lewis et al. (2007) were unable to achieve using radioactive isotopes. There was an overall decrease in rates of protein synthesis in gill, heart, muscle and liver under severe hypoxic conditions (5 and 10% air saturation). At 5 and 10% air saturation, protein synthesis rates decreased by 50–55% in gill, 58–72% in heart, 45–55% in muscle and 86–96% in liver. These results are similar to those of Lewis et al. (2007), who exposed oscars to 10% air saturation for 3 h and showed a decrease in protein synthesis of 50–60% in heart, gill and liver. Crucian carp (*Carassius carassius*) exposed to anoxic conditions for 48 h also showed a decrease in rates of protein synthesis by 95% in liver, 53% in heart and 56% in white muscle (Smith et al., 1996). A decrease in the rate of protein synthesis is a typical response to hypoxia exposure in tolerant species in order to reduce ATP demands and prolong survival (Lewis et al., 2007; Smith et al., 1996). The observed decrease in protein synthesis contributes to the metabolic rate depression during hypoxia, which was previously determined in oscars (Almeida-Val et al., 2000; Lewis et al., 2007; Muusze et al., 1998). The extent to which protein synthesis plays a role in the metabolic rate depression may be approximated. Using the fractional rate of protein synthesis in the tissues measured here and their relative mass with respect to whole-body mass (muscle 44%, liver 0.8%, gill 1.6% and heart 0.1%, T. MacCormack, personal communication), we estimate that the whole-body rate of protein synthesis decreases by 86% in the fish exposed to 5% air saturation. The cost of protein synthesis is not known in oscars; however, protein synthesis has been estimated to account for anywhere between 23 and 42% of the metabolic rate in other fish species (Carter and Houlihan, 2001; Houlihan et al., 1995). From this information, we estimate that the decrease in the rate of protein synthesis would account for a 20 to 36% decrease in metabolic rate. This is assuming that protein synthesis rates behave similarly in other tissues.

Although it is well established that protein synthesis rates decrease during hypoxia, the mechanisms controlling this decrease are not well documented in fish. In mammalian cell lines, protein synthesis rates are controlled via several signaling pathways, including mTOR, and the unfolded protein response (PERK) (Wouters and Koritzinsky, 2008). One recent study by Mohindra et al. (2016) exposed a hypoxia-tolerant species of catfish, *Clarias magur*, to near-anoxic conditions for 1–12 h and observed a decrease in transcript number of P70S6K in hypoxic compared with normoxic fish. This suggests a regulation of protein synthesis via mTOR, as P70S6K is one of mTOR's downstream targets (Jaeschke et al., 2002). However, mTOR regulates protein synthesis by post-translational modifications (phosphorylation) of its downstream targets; therefore, it is difficult to draw conclusions from transcript numbers. We looked at phosphorylation levels of 4EBP1, one of mTOR's major targets. We observed a significant decrease of 4EBP1 phosphorylation at 5 and 10% air saturation in the liver. There was also a drop of 4EBBP1 phosphorylation at 5 and 10% air saturation in the heart. These findings are consistent with signaling that leads to decreases in protein synthesis.

Another important signaling pathway in the hypoxia response of mammalian cells is the endoplasmic reticulum stress response. Again, this pathway has scarcely been studied in fish, and this is the first time that eIF2-α activation has been directly assessed in fish during hypoxia. Here we observed a substantial increase in the phosphorylation of eIF2-α in liver, at low air saturation levels, again consistent with decreases in rates of protein synthesis. One study on the common sole, *Solea solea*, showed that mRNA transcript numbers for a downstream target of eIF2-α, ATF4, increased during hypoxia (Mazurais et al., 2014). ATF4 is a transcription factor involved in protein folding and is activated by p-eIF2-α (Mazurais et al., 2014). We also attempted to measure eIF2-α phosphorylation in the heart, but we were not able to detect a signal (total protein and phosphorylated protein) by western blot. In a recent study on the human proteome, eIF2-α was also not detected in the heart when using specific antibodies (Uhlén et al., 2015).

We hypothesized that the rate of protein synthesis during hypoxia would decrease following the phosphorylation levels of 4EBP1 and eIF2-α. This is indeed the case at 10 and 5% air saturation; however, in the liver, we observed a decrease of protein synthesis at 20% air saturation without detecting any significant changes in the

phosphorylation levels of 4EBP1 and eIF2-α. This may suggest that other control mechanisms are involved in the regulation of protein synthesis during hypoxia. However, this observation may also be a result of the much lower resolution of the western blotting technique used to detect the phosphorylation levels of these signaling molecules.

Protein degradation

Both protein synthesis and degradation could potentially slow down during hypoxia (Leveelahti et al., 2011). The impact of hypoxia on protein degradation in the present study is not totally resolved and may have been influenced by the oscars not feeding for the experimental period that occurred 14 to 21 days post capture. In the present experiment, glycogen reserves were not utilized under the hypoxic conditions. This implies that the fish were utilizing onboard lipids or proteins as metabolic fuel to support the remaining aerobic metabolism. Hypoxia resulted in lipid utilization in liver in both oscars (De Boeck et al., 2013) and goby (*Gillichthys mirabilis*) (Gracey et al., 2001), presumably as a preferred metabolic fuel under hypometabolic conditions. In oscars, food deprivation for 10–14 days resulted in a decrease in liver lipid content and eliminated the difference between normoxic and hypoxic fish when expressed as mg g^{-1} tissue. The implication of this is that as the length of time of food deprivation increases, protein is called upon to a greater relative extent to support the reduced residual oxidative metabolism.

In the present experiments, absolute rates of NH_4 were in the same range as previously noted for normoxic oscars (Wood et al., 2007; Wood et al., 2009; De Boeck et al., 2013). Earlier studies all report a decrease in the rate of NH_4 under hypoxia (Wood et al., 2007; Wood et al., 2009; De Boeck et al., 2013). In contrast, we did not see a statistically significant decrease in NH_4 excretion, although the average rate under hypoxia was 60% of that of normoxia. We offer two explanations for the difference. Foremost, in previous studies, NH_4 production was determined on the same animals exposed to different levels of hypoxia. The present experimental design, in which NH_4 production was determined for different animals at different levels of hypoxia, may have missed true differences owing to extreme individual variability and small sample size. Secondly, the present experiment was conducted on animals that went without feeding for longer than in previously published work. It is possible that these animals were already in a hypometabolic state in which lipid reserves were exhausted and, consequently, aerobic respiration was supported by protein catabolism even under hypoxia. This issue remains to be resolved.

Information at the biochemical level also remains equivocal. An acute exposure to hypoxic conditions generally did not lead to statistically significant changes in the maximal activity of the 20S proteasome or levels of polyubiquitinated proteins in liver, gills, heart or muscle. Similarly, there was no change in activity levels of lysosomal cathepsins or calpains. Taken together, the data do not provide any compelling evidence that protein degradation in oscars is reduced under hypoxia as a cost-saving measure over the 8 h time course, which might be a result of small sample size. Other studies support a contrary scenario. In stickleback, *Gasterosteus aculeatus*, exposed to 24% air saturation for 48 h and in zebrafish exposed to 10% air saturation for 3 weeks there was a decrease in proteasome subunit transcript levels (Leveelahti et al., 2011; van der Meer et al., 2005), consistent with possible decreases in protein degradation resulting in decreased ATP utilization. Then again, our experiment was conducted on fish that refused to eat for 3 weeks, and we cannot rule out that the fish were possibly in a starvation state and mainly relied on protein as a metabolic fuel, thus hindering the effects of

hypoxia on protein degradation. The relationship between food deprivation and hypoxia was previously studied in oscars (De Boeck et al., 2013). In that study, after 10–14 days of starvation, oscars coped well with hypoxia, mainly by having a lower critical partial pressure of oxygen than the fed fish. These authors argued that after 2 weeks the fish were still in phase II of food deprivation, meaning that protein degradation was low and degradation of lipids fueled most bodily metabolism (see Wang et al., 2006). Because the rates of ammonia excretion measured in our experiment are comparable to those previously measured (De Boeck et al., 2013), it is reasonable to assume that the fish in the present experiment had not yet entered phase III, i.e. when lipids are depleted and animals rely solely on protein as a metabolic fuel. Although the period of starvation was longer in the present experiment than that of De Boeck et al. (2013), the mass of the fish was higher in the present study (260 g versus 60–90 g), and typically, larger fish are more resistant to food deprivation. The regulation of protein degradation during hypoxia is poorly understood and warrants further study. Many cellular responses of organisms occur within the first few minutes or hours of hypoxia exposure; however, some processes are only activated after long-term exposure (Trübenbach et al., 2014). It is possible that an acute exposure to hypoxia such as in the present study was not sufficient to induce changes in biochemical indices of protein degradation.

Conclusions

This study describes molecular mechanisms that are involved in regulating protein synthesis during hypoxia in fish. We demonstrate that in the oscar, like in mammalian cells, protein synthesis is regulated by hypoxia signaling pathways and does not decrease solely due to a decrease in ATP when oxygen becomes limiting. Instead, tissues actively decrease rates of protein synthesis in order to spare ATP for more vital processes. This physiological adaptation, in part, allows oscars to maintain stable levels of ATP in order to prolong survival in hypoxic conditions (Lewis et al., 2007). In contrast, we found no evidence of change in rates of protein degradation based on NH_4 excretion, indicators of the ubiquitin proteasome pathway, or activity of lysosomal and non-lysosomal proteolytic enzymes, suggesting that protein degradation was maintained or at least not highly downregulated. The rate of oxygen consumption of oscars at 6–10% air saturation is approximately 50% of that of normoxia (Lewis et al., 2007; Muusze et al., 1998). Using a highly sensitive stable isotope technique, we were able to measure rates of protein synthesis in muscle for the first time. With this information, it is calculated that approximately 50% of the decrease in oxygen consumption could be due to decreases in rates of protein synthesis. The remaining decrease in oxygen consumption would require a downregulation of other processes such as Na^+ regulation (Wood et al., 2009). Downregulation of energy demand may be especially important because anaerobic metabolism is not highly activated.

Protein metabolism is a relatively new and unexplored aspect in understanding hypoxia tolerance. Most recent trends in hypoxia studies are leaning towards microarray and gene sequencing technologies, which will help identify better targets for studying hypoxia tolerance. There is an important knowledge gap concerning the different degradation pathways that could contribute to the hypoxia response. In-depth studies on different pathways that regulate the hypoxia response in fish are warranted, especially effects of the hypoxia inducible pathway, which we did not assess. Future comparative studies of hypoxia-tolerant and -sensitive fish species would help identify useful targets for studying hypoxia tolerance.

Author contributions

Conceptualization: A.A.C., W.R.D., D.C., W.H., S.G.L.; Methodology: A.A.C., W.R.D., D.C., W.H., S.G.L.; Formal analysis: A.A.C., W.R.D., S.G.L.; Writing - original draft: A.A.C., W.R.D., S.G.L.; Writing - review & editing: A.A.C., W.R.D., D.C., W.H., V.M.A., A.L.V., S.G.L.; Funding acquisition: W.R.D., V.M.A., A.L.V., S.G.L.

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